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BASIC MECHANISMS OF OCCUPATIONAL LUNG DISEASE

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EFFECTS OF INHALED ENDOTOXIN-CONTAINING BACTERIA

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The various biologic responses that may be initiated by inhalation of ubiquitous, gram negative, endotoxin-containing bacteria will be examined. A number of sensitive means of evaluating both systemic and intrapulmonary responses to such an inhalation are available and have been applied to a variety of laboratory animal species. Microorganisms chosen for study were selected from isolates obtained from either aerosols from the work environment or from materials which compose particulate aerosols in the work environment. Aerosols were prepared from saline suspensions of each respective test microorganism and administered for brief periods (30-60 min) to unimmunized animals. At appropriate intervals after the aerosol, the respective measurements were taken.

Such brief aerosols of certain organisms cause a leukocytosis, an increase in arterial oxygen tension, and an exaggerated release of intrapulmonary lactic dehydrogenase (LDH) within 4 hrs after challenge. These small aerosols even exert enough effect to predispose the animal to a hemorrhagic necrosis of the lungs when followed in 24 hrs by an intravenous administration of the same microorganism, in effect a pulmonary Shwartzman reaction.

Measurement of intrapulmonary release of LDH is maximal at 4 hrs, but depends on the species of gram negative bacterium used. Two give especially exaggerated responses, Citrobacter freundii, isolated from the air of a swine confinement facility, and Erwinia herbicola, one of the most common bacteria found on cotton bracts and cotton cardroom dust. When such animals' pulmonary lavages are examined for cellular content the species of bacterium inhaled again determines the type and composition of the cellular infiltrate seen. There is no relationship between the cellular infiltrate and intrapulmonary LDH production.

The rabbit is the species of choice for pyrogen testing of endotoxin, but exhibits no response to 30' aerosols of E. coli. However, if a more sensitive means of measuring imbalance of temperature maintenance is used, brief aerosols of certain gram negative bacteria do upset the temperature control mechanism.

Long term consequences of immunologically induced injury were studied by in vitro studies of murine lymphocytes using purified lipopolysaccharide found that in vitro mitogenesis varied depending on the source of LPS. Such a characteristic could lead to an enhancement of the immune response to inhaled antigens.

It was concluded that since small amounts of endotoxin-containing bacteria could induce such a variety of potentially injurious effects via inhalation in a variety of laboratory animals, it would be of great importance to ascertain what affects inhaled endotoxin would have in humans in explaining the pathogenesis of inhalation diseases of occupational origin.

MANGANESE DIOXIDE INDUCES ALVEOLAR MACROPHAGE CHEMOTAXIS FOR NEUTROPHILS

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Workers exposed to airborne manganese dioxide (MnO_2) may develop pneumonia resistant to chemotherapy. Previous animal experiments have demonstrated that exposure to airborne MnO_2 induces an invasion of polymorphonuclear leukocytes (PMN) into the airways 4 to 12 hours after exposure.

Experiments were undertaken to further study the role of pulmonary alveolar macrophages (PAM) for the development of this response. Guinea pig lung lavage fluid was centrifuged and washed, and the cell suspension incubated on one side of a leukocyte migration chamber. The incubation fluid employed was Hanks medium, containing inactivated serum. MnO_2 (2 mg/ml) was incubated with the cells for different time periods. PMN from quinea pig blood were isolated and incubated on the other side of the chamber.

Observations of the free lung cells demonstrated that phagocytosis of the MnO_2 particles commenced immediately, and was virtually completed within 30 min. Cells from control animals caused small directional migration across the filter. Cells which had phagocytosed MnO_2 particles showed a significantly higher degree of PMN migration.

The data suggest that the chemical pneumonia in terms of PMN invasion into the lungs after MnO_2 exposure is caused by an activation of PAM with subsequent release of PMN recruiting factors.

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DEPOSITION AND TRANSLOCATION OF INHALED SILICA

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Crystalline quartz (silica, SO_2) is a highly fibrogenic dust which causes significant lung disease in occupationally exposed individuals. While this has been known for many years, numerous questions exist regarding the early particle-cell interactions which lead to the initial lesions of silicosis. Virtually no information is available on the sites of initial particle deposition and the time-related events of silica translocation to various pulmonary cells. Such information is essential to achieving an understanding of the basic mechanisms of silica-induced lung disease.

White rats were exposed to 100 mg/m^3 of aerosolized alpha-quartz for 3 hrs in inhalation chambers. Groups of animals were sacrificed at the following times after exposure: 0 (immediately after exposure), 6, 12, 24, 48 and 72 hrs and 10, 14, 24 and 42 days. The lungs of these animals were fixed _in situ_ for light and electron microscopy by vascular perfusion through the right ventricle. In addition, macrophages were recovered by bronchopulmonary lavage from groups of sham and silica-exposed animals at the time periods noted above.

Immediately after exposure, we found by quantitative backscatter electron imaging that there were approximately 17,000 silica particles per mm^2 of alveolar duct surface. Twenty-four hours after inhalation, only 3,000 particles/mm^2 remained on the duct surfaces. Transmission electron microscopy revealed the anatomic compartments to which silica crystals were translocated over a 14 day post-exposure period. Particles were found in alveolar Type I cells, in interstitial cells and connective tissue, and within alveolar macrophages. Thirty-five to 65% of the _in situ_ macrophages contained silica depending upon the time period studied. Approximately 10-20% of these silica-containing cells appeared necrotic, while only 1-4% of macrophages from unexposed animals showed ultrastructural alterations.

Using x-ray energy spectrometry, we compared the elemental content of lavaged macrophages from exposed and unexposed animals. This technique allowed us to determine the percentage of silica-exposed cells which exhibited silicon x-ray counts statistically significantly different ($p < .01$) from the non-specific background of the control (unexposed) population. The data showed that immediately after expsoure, 27% of the macrophages contained silica crystals. Twelve hours after exposure this percentage increased to 72%, and no significant differences in this level were detected until 42 days post-exposure at which time the percentage of silica positive cells returned to 28%. Additional evidence of ongoing particle clearance is shown where the percentage of cells with silicon x-ray counts greater than 3000 (cts/30 sec) peaks at 22% (12 hrs after exposure) and returns to 1% at 42 days.

We have studied the deposition and translocation of aerosolized silica crystals in the lungs of rats exposed for 3 hrs. Large numbers of particles

passed through the conducting airways and deposited on alveolar surfaces. Because silica crystals were translocated to macrophages and to Type I - epithelial cells, significantly fewer particles were present on alveolar surfaces 24 hrs post-exposure. At this time, high percentages of silica-containing macrophages were seen in the lavage and in situ, and these high levels of macrophage participation were maintained through 24 days post-exposure. Interestingly enough, macrophage populations were not perturbed beyond normal limits regarding cell number, viability, bacterial killing, O_2 consumption and enzyme release. There is ultrastructural evidence of macrophage deterioration, although the studies of lavaged populations were not sensitive enough to detect any changes. We propose that the events reported here are components of normal, steady-state clearance which is operative subsequent to inhalation of a sub-pathogenic dose of potentially toxic particulates.

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INTERACTIONS OF ASBESTOS AND POLYCYCLIC AROMATIC HYDROCARBONS (PAH) IN CARCINOGENESIS OF THE RESPIRATORY TRACT

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An association has been established between exposure to asbestos and the occurrence of cancers of the respiratory tract. The risk of bronchogenic carcinoma (70-90 fold increase) is substantial in asbestos workers who smoke, whereas a relatively small increase in neoplasms (2-4 fold) is observed in non-smoking workers. A striking synergistic effect on tumor induction also is observed when asbestos and polycyclic aromatic hydrocarbons (i.e. PAH, chemical carcinogens found in cigarette smoke) are inhaled or instilled intratracheally into rodents. Because studies in whole animals have failed to provide insight into possible mechanisms of syndergism, we have developed techniques for studying the interactions of asbestos and PAH with cells of the tracheobronchial epithelium in organ and monolayer culture. To determine the sequence of neoplastic events occurring in the respiratory tract after exposure to these agents, the radioactively tagged PAH, Benzo(a)pyrene (PB) was adsorbed by evaporation onto crocidolite fibers and precipitated on the epithelial surfaces of tracheal explants for 1 hour. Control tissues were exposed to asbestos alone. Thereafter, morphological changes in vitro were monitored at weekly intervals after preparation of tissues for light and electron microscopy. After 4 weeks, tracheal explants were grafted subcutaneously on syngeneic hamsters to document their tumorigenicity. Tumors, the majority of which were carcinomas, appeared in a dosage dependent fashion from implanted tissues exposed to asbestos and PAH, whereas neoplasms were not found in tracheas exposed to asbestos alone.

A medley of approaches were used to study the interactions of BP and asbestos with tracheal epithelial cells in vitro. Autoradiography and scintillation spectrometry showed the rapid transfer of BP from both chrysotile and crocidolite fibers into the interior of the cell. Fibers interacted with cellular membranes and were phagocytized by tracheal epithelial cells. When BP was coated onto both types of asbestos before its addition to cultures, 70% of the total BP introduced entered the cells with 1 hour; 50% remained intracellularly after 8 hours. In contrast, if identical amounts of BP were added to medium, an initial influx of 50% was observed and cells retained only 5% of the initial amount at 8 hours. The integrity of DNA was monitored by alkaline elution after exposure of tracheal cells to asbestos, BP and BP adsorbed to asbestos at various concentrations. Single-strand breakage was observed with use of BP, but neither chrysotile nor crocidolite alone. Moreover, BP-coated asbestos did not increase strand breakage in excess of the amounts induced with BP alone.

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After exposure to crocidolite asbestos, increased synthesis of DNA and polyamines and the development of squamous metaplasia are observed in the tracheal epithelium. Although asbestos alone appears to be non- or weakly carcinogenic to tracheal epithelial cells, it facilitates the transfer of PAH into the cell and induces proliferative alterations that might be intrinsic to the induction and/or development of tumors. Supported by grant 00888 from NIOSH and grant CA 23514 from NCI.

THE EFFECTS OF INHALED ASBESTOS ON PULMONARY MACROPHAGES: A MORPHOLOGICAL, FUNCTIONAL, AND BIOCHEMICAL STUDY

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Occupational exposure to asbestos clearly is associated with interstitial fibrotic lung disease. We are studying the initial cellular events following a brief asbestos exposure, in an animal inhalation model (Brody et al, Amer. Rev. Resp. Dis. 123:670, 1981). Following a one-hour exposure to chrysotile asbestos, translocation of fibers occurs through a variety of cell types, followed by development of lesions at alveolar duct bifurcations. In our inhalation model, we have studied the effects of chrysotile asbestos on populations of pulmonary macrophages exposed to the inhaled dust *in situ*. Subtle, but consistent changes occur in macrophages recovered by pulmonary lavage at several time periods following a 1 hour exposure. Morphological, functional and biochemical techniques have been utilized to document these early changes.

Fisher 344 rats were exposed, nose-only, to 16.5 mg/m^3 (respirable mass) of aerosolized chrysotile asbestos for one hour in an inhalation chamber. Equal numbers of animals were sham-exposed to air. Forty eight, 72 and 192 hours after exposure, pulmonary macrophages were recovered from animals by gentle lavage with Ca^{++} and Mg^{++}-free phosphate buffered saline. Cells were counted and viabilities determined using trypan-blue exclusion. One-half million cells per animal were then plated on Thermanox coverslips and placed in a CO_2 incubator for 45 minutes. These macrophage monolayers were either cultured for an additional 24 hours for biochemical studies, incubated with opsonized carbonyl iron particles for a phagocytosis assay, or prepared for quantitative ultrastructural studies.

No significant differences were found in cell numbers and viabilities of macrophages recovered from asbestos exposed and matched sham-exposed animals at the three time periods. The macrophage yields averaged 1.1×10^6 cells/6 lavages, while the viability of cells ranged from 91-97%.

Using light and electron microscopic techniques, we found that approximately 27% of the macrophages recovered after 48 hours contained asbestos particles, while only 10-15% of cells recovered after 8 days showed evidence of particle uptake.

We have established a morphologic model of macrophage injury. The results of preliminary experiments have suggested that macrophage toxicity correlates with a loss of surface ruffles and consequent smoothing of the cell membrane, following plating on plastic culture surfaces. In untreated cultured monolayers, ruffled macrophages (M^+) consistently comprised 70-80% of the population. We have increased the number of smooth-surfaced cells by incubating macrophages with crystalline silica, and in acidic culture conditions. Smooth macrophages (M^-) have a depressed phagocytic capacity in comparison to ruffled macrophages (M^+). Macrophages recovered from asbestos and sham exposed animals were plated on coverslips for 45 minutes and prepared for scanning and transmission electron microscopy. Companion monolayers were cultured with iron beads

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(.5-2 μm in diameter) for 1 hour. Secondary and backscatter imaging were utilized to quantitate iron uptake by individual cells. The results show that an increase in the percentage of smooth-surfaced macrophages from asbestos-exposed animals correlates with a decreased capacity of the lavaged cell population to phagocytize iron beads.

Biochemical studies were carried out on macrophages recovered from asbestos and sham-exposed rats. Cells were cultured for 24 hours and intra- and extracellular lysosomal enzymes were measured spectrophotometrically. We found increased intra- and extracellular levels of acid phosphatase, β-N-acetylglucosaminidase, and α-mannosidase in the cells from asbestos-exposed animals. These findings correlated with cytochemical experiments on asbestos and sham-exposed macrophages, where the chrysotile exposed cells displayed an increased degree of acid phosphatase staining.

A one-hour exposure to chrysotile asbestos causes subtle, but measurable changes in certain parameters of macrophage populations. Alterations in cell numbers and viability were not demonstrated. On the other hand, diminished phagocytic capacity correlated with increased numbers of smooth-surfaced cells, and increased enzyme activity was demonstrated in asbestos-exposed cells. Whether or not these changes play a role in the slowly progressing pathogenesis of asbestosis is a subject of further study.

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CELLULAR AND BIOCHEMICAL ACUTE ALVEOLAR RESPONSE TO MINERAL DUSTS IN RODENTS

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We studied the pulmonary response of rats to the intratracheal instillation of 0.5 mg of chrysotile, acid leached chrysotile, crocidolite and quartz; furthermore the effect of 5 mg of quartz was also studied. Dusts were suspended in 0.3 ml of saline, chrysotile was sonicated prior to the instillation, control rats were instillated with saline. Alveolar washing fluid and lungs were studied at 24 hours, 48 hours, 1, 2 and 4 weeks after instillation. Rats were anaesthetized by an intraperitoneal injection of Nembutal, then exsanguinated by sectionning the dorsal aorta. The lungs removed from the chest cavity were washed several times with a total of 50 ml of saline cell. Viability was immediately estimated by the Trypan blue dye exclusion test and cells were counted using a hemocytometer. Smears were stained with Wright Giemsa stain for differential numeration.

We noticed that about 10% of the total number of macrophages did not contain any particles. The total number of cells harvested from animals treated with 0.5 mg of dust increased by about 2 folds during the two days following the instillation, then came back to a value close to that obtained from control animals up to 4 weeks, except for animals instillated with quartz which exhibited a second increase at 4 weeks. The total number of cells from animals treated with 5 mg of quartz remained 3 folds higher than that from control animals during all the experiment.

The increased number of alveolar cells was due on the one hand partly to a moderate increase of the number of macrophages, on the other hand mostly to a recruitment of neutrophils in airways. This recuritment was associated with an increased protein content of the lavage fluid: during the first two days was noticed an important exsudation of serum proteins, as indicated by the albumin content of the lavage fluid, while a local synthesis was probably responsible for the "proteinosis" observed in the animals inoculated with 5 mg of quartz. The role played by the complement system in the chemotaxis of leukocytes and the release of elastase within the alveolar spaces are under investigation.

PREDICTION OF HAZARD FOR FIBROUS MATERIALS

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Prediction of whether exposure to a new fibrous material could give rise to pulmonary disease involves estimation of the fibres which would be retained in the lungs. Basic data showing how retention depends on fibre diameter and length has been obtained by comparing the size of fibres in dust samples taken at the Paakkila anthophyllite mine with the size of fibres recovered from postmortem lungs. This asbestos mine, now closed, was unique in that it met the requirements for this work, in particular, wide ranges of fibre diameter and length encompassing the respirable size limits and fibre durability in lung fluids.

Fibres were measured using transmission electron microscopy and a method combining magnetic alignment and light scattering.

Representative results are presented graphically in Figures 1, 2 and 3. Figure 1 shows the size distribution of the airborne dust. Figure 2 the size distribution of lung dust and Figure 3 the relative retention derived from the two distributions.

When a material has the same density as anthophyllite, the expected size distribution of the lung dust may be derived from Figure 3 and the measured size distribution of the material. If, for instance, the material of interest is Paakkila anthophyllite one uses Figure 3 and Figure 1 and obtains Figure 2. For application to other materials, Figure 3 is modified to allow for the influence of density on fibre deposition.

The method has been applied to South African amosite fibres in Transvaal mines. Good agreement was obtained between expected and observed size distributions of lung dust.

To estimate the potential health risks of a material the expected size distribution of the lung dust becomes the first term in a multiplicative formula. Two terms allow for its durability in lung fluids and expected concentration. A fourth term quantifying its pathogenicity is derived from data available in the numerous 'in vivo', in vitro and epidemiological studies made on fibres differing in sizae and chemical composition. The product of the terms indicates the magnitude of the hazard.

Figure 1.

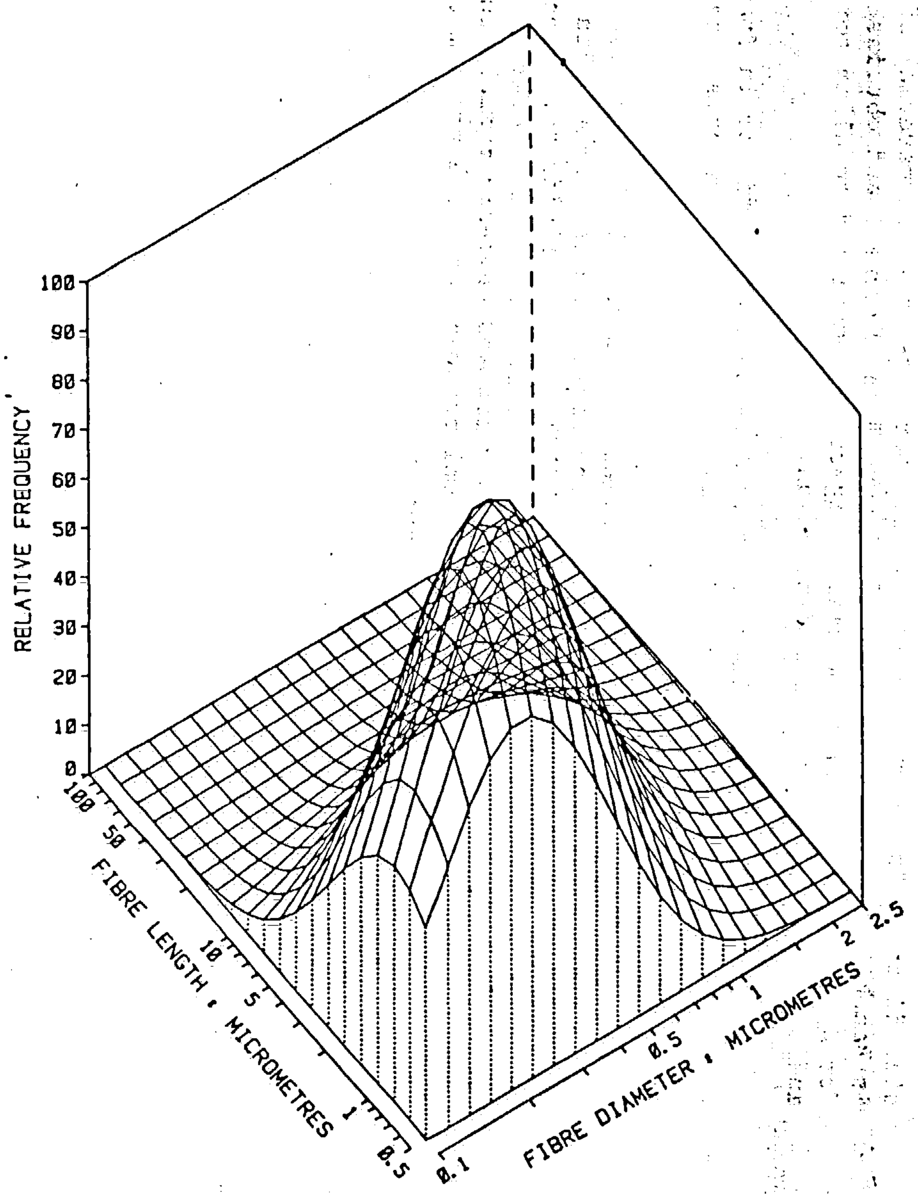

Figure 2

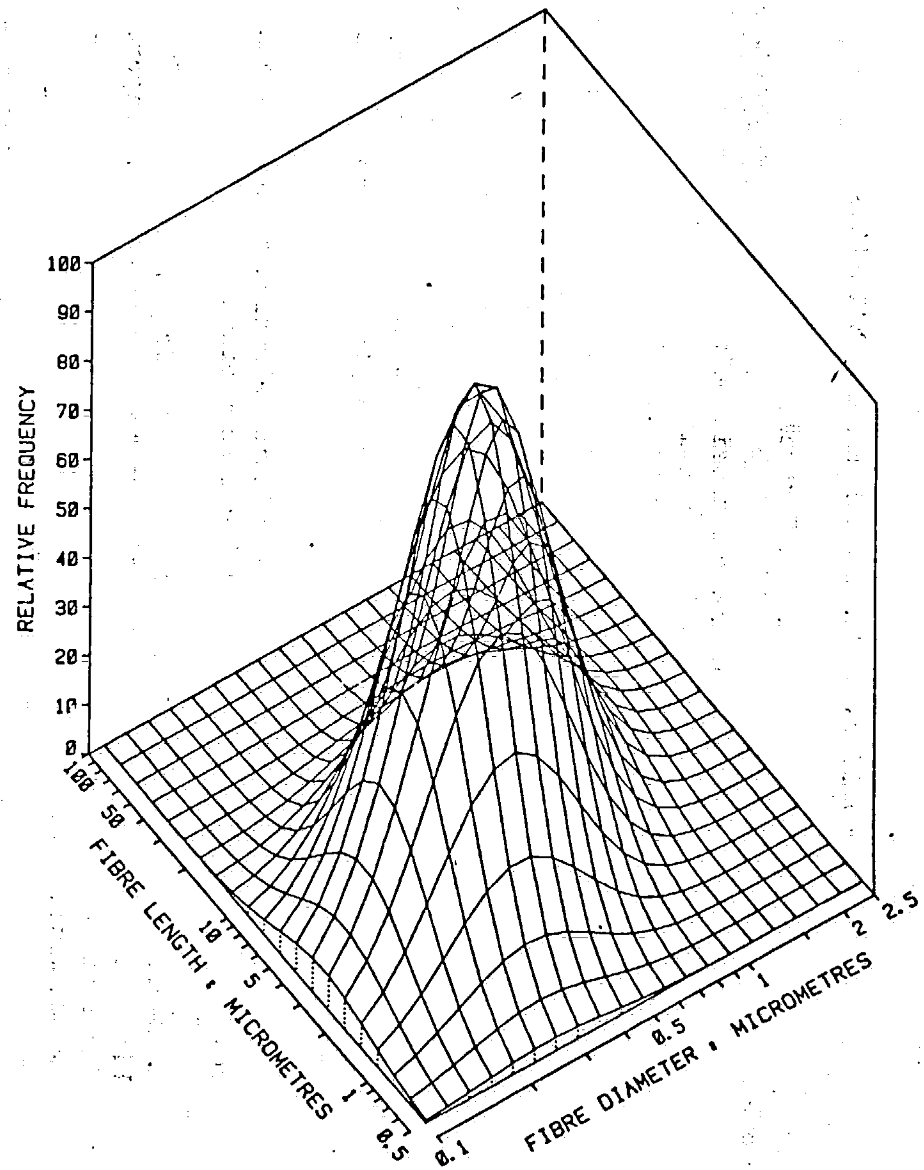

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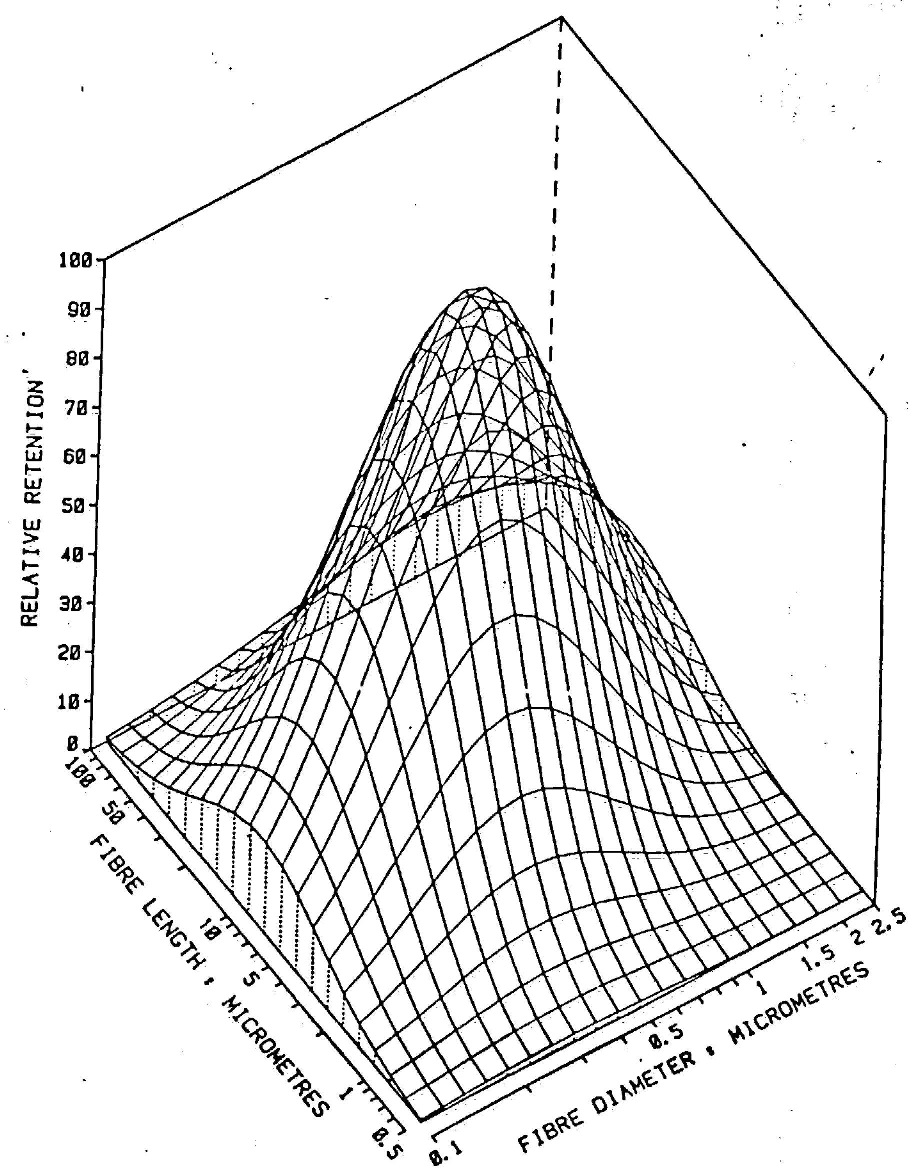
Figure 3


RELATIVE RETENTION'
100
90
80
70
60
50
40
30
20
10
0
FIBRE LENGTH : MICROMETRES
100
50
10
5
1
0.5
FIBRE DIAMETER : MICROMETRES
0.1
0.5
1
1.5
2
2.5

CORE COMPOSITION OF FERRUGINOUS BODIES FROM FORMER ASBESTOS WORKERS

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The terms "ferruginous body" and "asbestos body" are often used interchangeably. However, the potential for various non-asbestos entities such as glass fibers, fibrous aluminum silicates or silicon carbide forming the core of such bodies was established in animal studies. This awareness has led to use of the term pseudoasbestos bodies when the structure is not clearly on an asbestos nucleus and use of the nonspecific term "ferruginous body" unless core analysis has been achieved.

The use of electron microscopy for core identification has been recognized and has been correlated with several methods for collecting ferruginous bodies from tissue. The problems recognized in all previous methodologies consists of a lack of exposed core material in a large number of the bodies. This morphological configuration prohibits the use of either electron diffraction or x-ray energy dispersive analysis due to interference of the coating material.

A method developed in our facility permits selective removal of the ferruginous coat and therefore permits analysis of the core material.

The application of this technique in the present study was used to determine what, if any, entities other than asbestos would be found in ferruginous bodies from former amosite asbestos workers and to determine what distinguishing physical features might characterize the core material.

The tissue selected for digestion was from formalin fixed lung parenchyma of six occupationally exposed individuals. The presence of abundant ferruginous bodies had been confirmed in previous digestions as well as by review of tissue sections. A small portion (0.2 gram) of wet lung parenchyma was dissected and placed in 4 ml of previously filtered (through 0.2μ Nuclepore [Nuclepore Corporation, Pleasanton, CA] filter) bleach ("Wright" bleach - 9.2% Sodium Hypochlorite). Each sample was permitted to dissolve for thirty minutes at room temperature. Following gentle agitation, the solution was divided into three equal portions; each was immediately filtered through a 0.2μ Nuclepore filter. The collected material was washed by passing filtered distilled water through the system and the filters were allowed to dry.

Individual bodies from the filters were removed through micro-manipulation under the dissecting microscope and placed in geometrically defined areas on a clean Nuclepore filter. While a few larger ($\geq$ 100μ) and smaller ($\leq$ 15μ) bodies were included in the preparation, the average size body was 35μ. The 35μ range was consistent with size commonly found in the digestion of the tissue. Before further treatment, the ferruginous bodies marked on the filters were viewed and recorded in a Zeiss Photo-Microscope III.

The individual filters were respectively treated for four hours with a 40 ml wash of 8% oxalic acid in water baths maintained at 75°C. At the

end of the treatment interval, the filters were washed with 100 ml of previously filtered distilled water of the same temperature as that of the respective acid bath. The filters were dried, mounted on graphite stubs and studied in an AMR 1000A scanning electron microscope equipped with a United Scientific 30 mm^2 energy dispersive x-ray detector interfaced to a Tracor Northern TN-1710 analyzer.

The coating was removed from 442 isolated ferruginous bodies and the core material was determined to be amosite asbestos consistent with that used in the worker's previous industrial setting. While length has been reported to play a determining role in which fibers become coated, other distinguishing traits including surface irregularities, consisting of etching, fractures, fraying and complexities involving multifibers or fibrillar composition were commonly found. The majority of the uncoated fibers were shorter in length, and also had much smoother surface features with much fewer irregularities than those forming the nucleus of the ferruginous bodies.

THE EFFECT OF EXERCISE ON THE REGIONAL DISTRIBUTION OF INHALED PARTICLES

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We have investigated the deposition of an inhaled radioaerosol during exercise to determine if the increased ventilation of exercise has an important influence on the regional distribution of inhaled particles. Eight healthy volunteers and five normal cigarette smokers inhaled a submicron aerosol of ^{99m}Technetium sulphur colloid whilst sitting quietly at rest and again during exercise sufficient to produce a predicted oxygen uptake of 2 liters/min. Tracheobronchial and alveolar deposition were distinguished by performing aerosol imaging immediately and again 24 hours after each study, by which time particles deposited in the conducting airways had been cleared by mucocillary action. Regional changes were studied by dividing the right lung into three concentric zones: central, intermediate and peripheral, and three vertical zones: upper, middle and lower.

The increase in total particle deposition on exercise was similar to the increase in minute ventilation in both smokers and nonsmokers. There was a gradient in alveolar ventilation in the upright position at rest which increased from apex to base and largely disappeared on exercise. Alveolar ventilation in the upper zones on exercise was significantly less in smokers compared to nonsmokers. The proportion of particles depositing in the tracheobronchial region increased on exercise, particularly in smokers, with greatest deposition in the upper zones. The relative increase in particle deposition which occurs in the upper zones on exercise and the difference between smokers and nonsmokers may be important in the pathogenesis of occupationally related dust-induced lung disease.

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EVIDENCE AGAINST HAPTEN SPECIFICITY OF IgE ANTIBODIES TO ISOCYANATE PROTEIN CONJUGATES

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Reaginic antibodies have been detected in the serum of some TDI reactive workers by radioallergosorbent testing (RAST) with p-tolyl(mono)-isocyanate (TMI) conjugated to human serum albumin (HSA), leading to the proposal that TDI asthma may be IgE mediated. Studies from our laboratory and others have shown TMI-HSA specific IgE antibodies in sera of only a small proportion of workers, proven reactive by provocative inhalation challenge. Further, sera from a small percentage of asymptomatic workers also have TMI-HSA specific IgE antibodies.

Using a panel of 5 TMI-HSA RAST positive sera, we have shown cross-reactivity with methylene bisphenyl diisocyanate (MDI), hexamethylene diisocyanate (HDI), xylene diisocyanate (XDI), and monomeric and trimeric isophorone diisocyanate (IPDI) conjugated to HSA. Inhibition studies with these conjugates indicated lack of hapten specificity, raising the possibility that the antibodies might be carrier specific. RAST using TMI, MDI, HDI and XDI conjugated to non-human protein carriers were negative, supporting this hypothesis. Further evidence for carrier specificity was obtained by positive RAST results with HSA-conjugated to acetic anhydride which, like isocyanates, is thought to react with epsilon amino groups of lysine.

These results suggest that inhalation of isocyanate vapors may cause in vivo modification of human proteins which might, in certain individuals, confer antigenicity. This may play an important role in the disease pathogenesis.

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